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Exploring virulence of new and less studied species of *Metarhizium* spp. from Brazil for two-spotted spider mite control

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Abstract The two-spotted spider mite *Tetranychus urticae* is an important pest of strawberry crops in Brazil and many other countries. Focus for biocontrol studies involving entomopathogenic fungi has been on three species from the genus *Metarhizium*: *M. anisopliae* sensu stricto (s.s.), *M. brunneum* and *M. robertsii*. Also, the species *Beauveria bassiana* has been studied for spider mite control and one isolate (ESALQPL63) is commercially available in Brazil. New and undescribed *Metarhizium* species have been found recently in Brazil and provide a pool of isolates with potential for biocontrol in Brazil and probably also elsewhere. The mortality of adult females of *T. urticae* when exposed to four new Brazilian species of *Metarhizium* was compared to the mortality when exposed to *M. anisopliae* s.s., *M. brunneum*, *M. pingshaense*, *M. robertsii* and *Beauveria bassiana* ESALQPL63. Fungal suspensions were sprayed onto mites at 10^7 conidia/mL with 0.05% Tween 80 in laboratory bio-assays. We measured total mortality and percentage sporulating cadavers 10 days after exposure and calculated median lethal time (LT_{50}). The lowest LT_{50} (4.0 ± 0.17) was observed for mites treated with *Metarhizium* sp. Indet. 1 (ESALQ1638), which also performed well with respect to mortality after 10 days and capacity to sporulate from cadavers. Among the other little studied species tested, *M. pingshaense* (ESALQ3069 and ESALQ3222) and *Metarhizium* Indet. 2 (ESALQ1476) performed well and were comparable to *B. bassiana* (ESALQPL63). The new *Metarhizium* isolates and species thus showed potential for biological control.

Keywords *Tetranychus urticae* · Biocontrol · Virulence · Entomopathogenic fungus

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Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is one of the main pests of horticultural crops worldwide (Attia et al. 2013), and it is a main pest of strawberry crops in Brazil (Iwassaki et al. 2015). This spider mite attack affects these crops by feeding on the undersurface of leaves, causing curling and discoloration, which lead to reduced photosynthetic activity and leaf abscission (Attia et al. 2013; de Moraes and Flechtmann 2008). Furthermore, *T. urticae* has developed resistance to many chemical pesticides (Nicastro et al. 2010; Stumpf and Nauen 2001). According to Wu et al. (2016), widespread insecticide/acaricide usage has significantly reduced the number of the mites' natural enemies in crops, thus allowing *T. urticae* populations in the crops to grow.

One alternative to chemical pesticides is biological control with entomopathogenic fungi, in particular species from the phylum Ascomycota. Entomopathogenic fungi from the genus *Metarhizium* are produced and marketed for biological control of various pests (Faria and Wraight 2007; Vega et al. 2012). Maniania et al. (2008) highlighted the importance of testing more isolates of entomopathogenic fungi to evaluate whether among the tested isolates were some with high potential for biocontrol. Whereas some species from the genus *Metarhizium* (*M. anisopliae* s.s., *M. brunneum*, and *M. robertsii*) have been subjected to several studies about virulence and other features, other described species from that genus (for example *M. pingshaense*) are more or less unknown with respect to biological properties. Also, new *Metarhizium* species have recently been discovered in Brazil (Rezende et al. 2015), so far referred to as *Metarhizium* Indet. 1, 2, 3, 4 and 5.

In this study, we aimed to compare the virulence (as measured by mortality) to *T. urticae* of 14 Brazilian *Metarhizium* isolates. We included *Metarhizium* Indet. 1, 2, 4 and 5, *M. pingshaense*, *M. anisoliae* s.s., *M. brunneum*, and *M. robertsii* and used a commercial isolate of *Beauveria bassiana* as a comparison. Also, the ability to sporulate from infected and killed mites was measured.

Materials and methods

Fungal origin

Two isolates each were selected of *M. robertsii*, *M. anisopliae* s.s., *M. brunneum*, *M. pingshaense*, and one isolate each of *Metarhizium* Indet. sp. 2 and 5 obtained from strawberry crop soils in southern Minas Gerais State in Brazil between 2012 and 2013 (Table 1) from the Collection of Entomopathogenic Microorganisms of ESALQ-USP. These were compared to two isolates each of *Metarhizium* sp. Indet. 1 and 4 (A.B.R. Zanardo unpubl. data) from the same collection. *Beauveria bassiana* (ESALQPL63), commercially available in Brazil for the control of this pest, was used as control. All fungi used were molecularly identified using TEF marker as suggested by Bischoff et al. (2009).

Tetranychus urticae stock colony

Tetranychus urticae was reared on Jack bean plants *Canavalia ensiformis* (Fabaceae) in a room at 25 °C, 60% RH, and 12 h photoperiod at the University of São Paulo

Table 1 Origin of *Metarhizium* spp. and *Beauveria bassiana* isolates and median lethal time ($LT_{50} \pm SE$) of *Tetranychus urticae* mites sprayed with each isolate

Collection no. (ESALQ)	Species	Crop, source and method for isolation[a]	Locality[b]	$LT_{50} \pm SE$	95% CI[c]
3180	*M. robertsii*	IB: Strawberry soil	Estiva/MG	5 ± 0.28	(4.45–5.55)
2693	*M. robertsii*	SM: Strawberry rhizosphere	Inconfidentes/MG	5 ± 0.18	(4.65–5.35)
2651	*M. anisopliae*	IB: Strawberry soil	Cambuí/MG	5 ± 0.28	(4.46–5.54)
3054	*M. anisopliae*	SM: Strawberry rhizosphere	Inconfidentes/MG	5 ± 0.23	(4.55–5.45)
3093	*M. brunneum*	IB: Strawberry soil	Senador Amaral/MG	5 ± 0.24	(4.52–5.48)
2623	*M. brunneum*	IB: Strawberry soil	Inconfidentes/MG	7 ± 0.83	(5.38–8.62)
3222	*M. pingshaense*	IB: Strawberry soil	Estiva/MG	5 ± 0.27	(4.48–5.52)
3069	*M. pingshaense*	IB: Strawberry soil	Cambuí/MG	5 ± 0.15	(4.70–5.30)
1638	*Metarhizium* sp. Indet.1*	IB: Savanna soil	Rio Verde/GO	4 ± 0.17	(3.68–4.32)
1608	*Metarhizium* sp. Indet.1*	IB: Savanna soil	Rio Verde/GO	5 ± 0.15	(4.70–5.30)
1476	*Metarhizium* sp. Indet.2*	IB: Strawberry soil	Cambuí/MG	5 ± 0.23	(4.54–5.46)
1684	*Metarhizium* sp. Indet.4*	SM: Sugarcane rhizosphere	Iracemapolis/SP	6 ± 0.48	(5.06–6.94)
1660	*Metarhizium* sp. Indet.4*	IB: Sugarcane soil	Piracicaba/SP	8 ± 1.49	(5.08–10.91)
3140	*Metarhizium* sp. Indet.5*	IB: Sugarcane soil	Cambuí/MG	6 ± 0.34	(5.33–6.67)
PL63	*Beauveria bassiana*	Insect, *Atta* sp.	Piracicaba/SP	5 ± 0.16	(4.69–5.31)

*New M*etarhizium* spp. not yet taxonomically described and named

[a]Isolation method: *IB* insect baiting, *SM* selective agar medium, [b]Brazilian states: *MG* Minas Gerais, *GO* Goiás, *SP* São Paulo, [c]95% confidence interval of LT_{50}

(ESALQ-USP) since 2014. The plants were irrigated 3 × a week. Old and highly infested plants were replaced by new ones as required.

Fungal production

All selected isolates were grown in Petri dishes (90 × 15 mm) at 25 ± 1 °C on PDA medium (Potato Dextrose Agar, Difco™, USA). Conidia were harvested by scraping the surface of 2-week-old sporulating cultures and then suspended in 5 mL sterile distilled water containing 0.05% Tween 80 (Oxiteno, São Paulo, Brazil) in a 40-mL flat bottom glass tube (stock suspension). The conidial suspension was vortexed for 1 min to produce a homogenous conidial suspension and counted on Neubauer chamber (K5-0111 model, KASVI, Brazil) to obtain a 10^7 conidia/mL suspension of each isolate.

Bioassay

Petri Dishes (3.5 cm diameter, 1.5 cm high) were filled with 3 mL of 0.5% water agar and a Jack Bean disc leaf (2 cm diameter) was placed on top of the agar with the abaxial surface up. Then, 12 *T. urticae* females were transferred with a fine paintbrush to the center of the leaf disc. Each Petri dish was sprayed with 1 mL of a 10^7 viable conidia/mL suspension of one isolate with 0.05% Tween 80 using a Potter Spray Tower (Burkard Manufacturing, Rickmansworth, UK). The device was adjusted to a pressure of 0.7 psi, with a coverage of 1.5 mg/cm^2. To avoid contamination between treatments, the sprayer was cleaned with alcohol and washed 3 × with distilled water, and the first spray of each treatment was discarded. A control with distilled water plus 0.05% Tween 80 was included. Five replicates (Petri dishes) were sprayed separately with each concentration. The entire experiment was repeated 3 × yielding 180 mites per treatment; in total 2880 mites were used in the entire experiment. The data from the three experiments were analyzed together.

Mortality was recorded daily for 10 days. Dead mites were individually placed in 24-cell well culture plates with a lid on, containing moistened cotton with sterile distilled water to ensure conditions of high relative humidity to allow fungus growth on the surface of the cadaver. Mortality caused by fungus was confirmed by scraping the surface of the mite with a sterile loop to collect the fungi and microscopic examination of this material.

Statistical analysis

The survival parameters of infected mites were analyzed using the Kaplan–Meier survival analysis using Log Rank (Mantel–Cox) test in v. 22.0.0 SPSS (2013). Quasi-binomial models were fitted to the total mortality and sporulated cadaver's data separately; F-tests were performed to assess significance of effects using a GLM procedure (multcomp R package) through ANOVA. Treatment differences were tested using 95% confidence intervals using Tukey HSD contrasts. Analyses were performed using the R statistical software environment (R Development Core Team 2015).

Results

All *Metarhizium* isolates were pathogenic to *T. urticae* and mortality of mites differed significantly between untreated control and fungus treated specimens in the survival

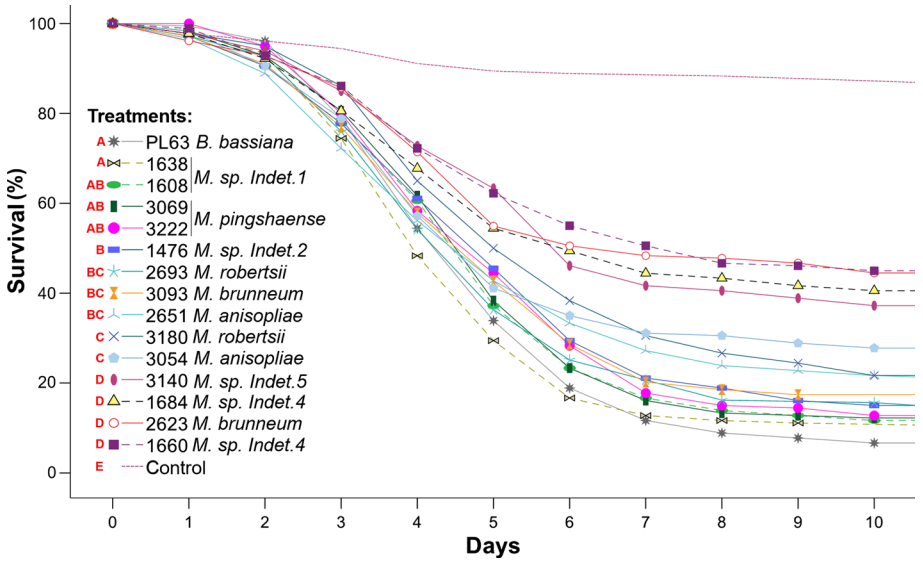

Fig. 1 Survival curves of *Tetranychus urticae* (n = 180 mites per treatment) inoculated with *Metarhizium* spp. The survival curve refers to the percentage of mites surviving up to 10 days post-treatment. Different letters indicate differences among treatments [Log Rank (Mantel–Cox) $p < 0.05$]

analysis (Fig. 1). The survival curve of the mites treated with our control isolate, *B. bassiana* ESALQPL63, was similar to *Metarhizium* sp. Indet. 1 (ESALQ1608 and ESALQ1638), and *M. pingshaense* (ESALQ3069 and ESALQ3222) but differed from all other isolates [Log Rank (Mantel–Cox) $p < 0.05$], confirming that *Metarhizium* sp. Indet. 1 and *M. pingshaense* were as efficient as *B. bassiana*. *Metarhizium* sp. Indet. 4 (ESALQ1660 and ESALQ1684), *M. brunneum* (ESALQ2623) and *Metarhizium* sp. Indet. 5 (ESALQ3140) were all pathogenic, but showed a lower mortality on the exposed mites than the other isolates.

The only isolate standing out with respect to low LT_{50} was *Metarhizium* sp. Indet. 1 ESALQ1638 (Table 1): 4.0 ± 0.2 days (95% CI 3.7–4.3). In the other end of the range, mites treated with *Metarhizium* sp. Indet. 4 ESALQ1660 had a LT_{50} of 8.0 ± 1.5 (95% CI 5.1–10.9).

Mortality after 10 days differed significantly between untreated control and fungus treated specimens (Fig. 2). The mortality of mites treated with *B. bassiana* ESALQPL63 showed a mortality of 93.0 ± 1.0% 10 days after exposure and was similar to that of eight other isolates. *Beauveria bassiana* ESALQPL63 and *Metarhizium* sp. Indet. 1 (ESALQ1608 and ESALQ1638), differed ($F_{1,15} = 29.92$; $p < 0.001$) from *M. anisopliae* s.s. (ESALQ2651 and ESALQ3054), *M. brunneum* (ESALQ2623), *Metarhizium* sp. Indet. 4 (ESALQ1660 and ESALQ1684) (56 ± 9.7 and 59 ± 2.8%) and *Metarhizium* sp. Indet. 5 (ESALQ3140), the isolates resulting in lowest mite mortalities.

Sporulation was observed in approximately 80% of spider mite cadavers in all treatments. *Beauveria bassiana* (ESALQPL63), *Metarhizium* sp. Indet. 1 (ESALQ1608 and ESALQ1638), and *Metarhizium pingshaense* (ESALQ3222) showed the highest percentage of sporulation ($F_{1,15} = 67.45$, $p < 0.001$) compared with *M. anisopliae* s.s. (ESALQ2651 and ESALQ3054), *M. brunneum* (ESALQ2623), *Metarhizium* sp.

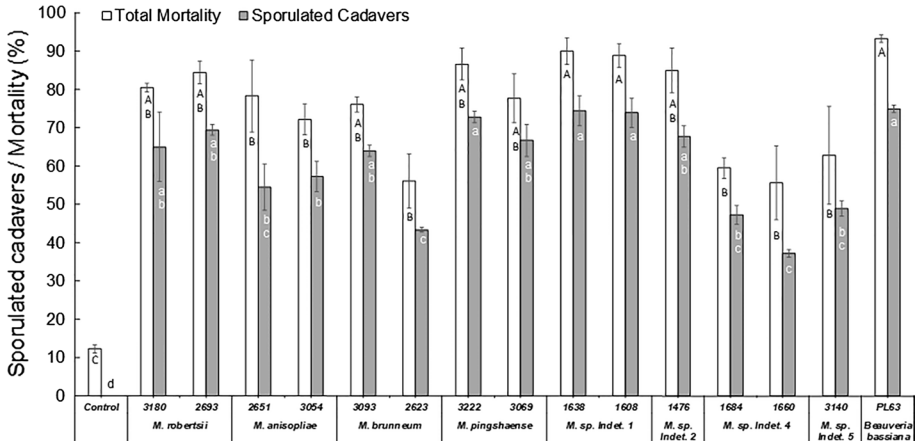

Fig. 2 Mortality and sporulation from cadavers of *Tetranychus urticae* females at 25 °C and 12:12 L:D 10 days after being sprayed with 14 isolates of *Metarhizium* spp. or *Beauveria bassiana* (isolate PL 63) with 0.05% Tween-80. Different lower-case letters indicate treatment differences in sporulated cadavers; different upper-case letters indicate treatment differences in total mortality by quasi-binomial models and F-tests through ANOVA and Tukey HSD comparison ($p < 0.05$)

Indet. 4 [ESALQ1660 (37 ± 1.0%) and ESALQ1684] and *Metarhizium* sp. Indet. 5 (ESALQ3140).

Discussion

This study revealed for the first time the potential of new and less studied species and isolates of *Metarhizium* as biocontrol agents of *T. urticae*. The mortality levels obtained in our study were in an overall sense similar to other studies using *Metarhizium* species against spider mites elsewhere. Chandler et al. (2005) showed reductions of all developmental stages (eggs, nymphs and adults) of *T. urticae* when *M. anisopliae* s.l. was applied; they also studied the efficacy of Naturalis-L (*B. bassiana*-based mycopesticide—Troy Biosciences, Phoenix, TX, USA) which reduced *T. urticae* populations by 97%. Up to 93 and 96% reductions in *T. urticae* population density on cucumber and tomato, respectively, were observed following application of Naturalis-L (Marcic et al. 2012). Bugeme et al. (2015) reported that *M. anisopliae* s.l. formulations were as effective as abamectin (chemical insecticide) in reducing *T. urticae* densities on common bean in both screenhouse and field experiments. An emulsifiable formulation of *M. anisopliae* s.l. was tested in the control of five Tetranychidae species including *T. urticae*, in irrigated cotton fields in a desert area in the Tarim Basin of northwestern China (Shi et al. 2008). A high potential for practical use in the management of the spider mites using this technique was reported by the authors.

Based on the 2009 classification of *Metarhizium*, the fungus previous known as *M. anisopliae* s.l. actually comprises complex of nine species: *M. anisopliae* s.s., *M. guizhouense*, *M. pingshaense*, *M. acridum*, *M. majus*, *M. lepidiotae*, *M. brunneum*, *M. globosum* and *M. robertsii* (Bischoff et al. 2009), so in literature before this year the name '*Metarhizium anisopliae*' may refer to any of these species. In our studies, we had for the

first time a possibility to compare virulence between eight different and partly unknown *Metarhizium* species determined by molecular methods, and have by that a solid background for choosing one or more isolate for further studies.

Although all evaluated fungal isolates were able to infect adult female of *T. urticae* mite in laboratory, there were significant variations amongst the isolates. These results revealed the potential of the unassigned new lineages of *Metarhizium* as biocontrol agents. *Metarhizium* sp. Indet. 1 ESALQ1638 stands out (based on survival curves of treated hosts, high mortality and sporulation on cadavers 10 days after exposure, low LT_{50}, and sporulation) when compared with the other tested *Metarhizium* isolates, although differences were not big. *Metarhizium* sp. Indet. 1 was therefore in the same range as the isolate registered for control of mites, *B. bassiana* (ESALQPL63).

Metarhizium sp. Indet. 1 and the other new species of *Metarhizium* are here for the first time studied for their potential. Also, *M. pingshaense* is largely underexplored. In one study, an isolate of *M. pingshaense* (MGC02) caused more than 80% larval mortality of *Anomala cincta* (Coleoptera: Scarabeidae) in laboratory assays (Guzmán-Franco et al. 2011). Pena-Pena et al. (2015) reported that the same isolate was potentially useful in controlling root-feeding pests by seed inoculation, confirming its ability to endophytic colonize maize roots.

Subsequent studies may include more biological data in an exploration, if for example *Metarhizium* sp. Indet. 1 or *M. pingshaense* possess characters allowing them to be superior to *B. bassiana*, *M. anisopliae*, *M. brunneum* or *M. robertsii* for mite control. Data on sporulation, as in Fig. 2, are for example important as these data reveal information of the potential of the fungi to reproduce after application and by that infect more targets over time.

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